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Reconstitution of active sucrose transport in plant proteoliposomes

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The proteins of purified plasma membranes from sugar beet (*Beta vulgaris* L.) leaf were solubilized and separated on a size exclusion column. The fractions eluted from the column were monitored by ELISA with antibodies directed to a putative sucrose carrier protein. The peak most reactive in ELISA was approximately 120 kDa, and yielded a 40 kDa peak after denaturation by SDS. The 120-kDa peak was recovered and used for reconstitution experiments with asolectin. Upon imposition of an artificial pH gradient and electrical gradient, the obtained proteoliposomes exhibited active transport of sucrose, but not of valine. The active transport of sucrose was inhibited by *N*-ethylmaleimide and $HgCl_2$.

Sugar transport; Reconstitution; Sucrose; Plant plasma membrane; *Beta vulgaris* L.

1. INTRODUCTION

Plant productivity depends not only on photosynthesis, but on the ability of the plant to export sucrose towards the organs harvested for human consumption [1]. In many crops, sucrose is the major form of sugar transport, and has to cross several plasma membranes between its site of synthesis, i.e. the cytoplasm of the assimilating cell, and its site of storage, i.e. the vacuole of a storage root cell or of a fruit cell. Uptake of sucrose across the plasma membrane involves co-transport with protons, and is powered by a proton-pumping ATPase [2,3]. This ATPase has been purified, reconstituted and cloned [4,5]. On the other hand, the proteins mediating sucrose transport are still poorly known. A 62-kDa [6] and a 42-kDa polypeptide [7,8] have been proposed as putative sucrose carriers. In both cases, the approach used was indirect (photoaffinity labeling, [6]) and differential labeling [7] combined to the use of polyclonal antibodies [8]. Recently, methods were designed to study the active transport of sucrose across purified plasma membrane vesicles artificially energized by a proton motive force [9–13]. However, up to now, no technique allowing the functional reconstitution of the carrier is available. This technique would obviously be helpful to identify the sucrose carrier, and it might also be useful for the identification of other carriers.

Abbreviations: CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate; DTT, dithiothreitol; NEM, *N*-ethylmaleimide

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The present report takes benefit of our previous identification of a sucrose carrier protein and presents the first reconstitution data on a proton-driven sucrose uptake system in artificial membranes. Although antibodies directed to the 42-kDa plasma membrane polypeptide assumed to be the sucrose carrier were available, their amount was quite limited and the preparation of the antigen needed for immunization is very long. Therefore, immunoaffinity chromatography of the 42 kDa polypeptide was not possible, and an alternative strategy was developed to identify membrane fractions competent in sucrose transport.

2. MATERIALS AND METHODS

2.1. *Plant material*

Sugar beet plants (*Beta vulgaris* L. var. Aramis) were grown as described previously [7]. Mature exporting leaves (4–5 weeks old) were used for the preparation of plasma membrane vesicles. Plasma membranes vesicles (95% pure) were obtained from a microsomal fraction by two-phase partitioning between Dextran T 500 and polyethylene glycol 3350 ([7,14]).

2.2. *Solubilization and high-performance liquid chromatography*

Plasma membrane vesicles were resuspended (4 mg/ml) in a medium containing 20 mM potassium phosphate (pH 6.0), 330 mM sorbitol, 10% glycerol, 0.5 mM $CaCl_2$, and 0.25 mM $MgCl_2$. CHAPS (final concentration 1%) was added dropwise under constant stirring at 4°C from a 10% stock solution prepared with the same medium. After 45 min solubilization, the insoluble material was precipitated by centrifugation at 100 000 × *g* for 30 min. The supernatant (referred to as 'CHAPS supernatant') was frozen at −70°C until further use. After thawing in a water bath at 25°C, the solubilized proteins (1.5 mg, 500 μl) were applied to a TSK SW-3000 gel filtration column (30 × 0.75 cm, Supelco) equilibrated with 50 mM Tris-acetate (pH 6.7), 100 mM NaCl and 0.1% CHAPS. The proteins were eluted at a flow rate of 0.4 ml/min and collected in 0.4 ml aliquots. The ability of each fraction to react with an antiserum directed against the 42-kDa polypeptide of the plasma membrane assumed to be the sucrose carrier [7,8], was tested by ELISA as described below.

The gel filtration column was calibrated with molecular weight standards, in the presence of 0.1% CHAPS when the 'CHAPS supernatant' was analyzed, or in the presence of 0.1% SDS when the proteins denatured by SDS were analyzed. The molecular weight standards were obtained from Boehringer (Combithek size II) and consisted of cytochrome *c* (12.5 kDa), chymotrypsinogen A (25 kDa), hen egg albumin (45 kDa), bovine serum albumin (68 kDa) and aldolase (158 kDa).

2.3. *Immunological procedures*

The 42-kDa antigen was prepared from preparative SDS-PAGE of purified plasma membranes from sugar beet leaves [8]. A mouse ascites fluid directed against this polypeptide was obtained by the immunization procedure described in [15]. These antibodies were used to monitor the reactivity of the plasma membrane proteins eluted from the gel filtration column by means of an ELISA test.

Two hundred μl (3–7 μg protein) of each aliquot recovered from HPLC were used to coat ELISA plates overnight. This amount of protein was higher than that needed to saturate the adsorption sites of the well (2 μg). The wells were washed three times for 15 min with phosphate buffered saline containing 3% defatted dry milk and 0.5% (v/v) Tween 20. The ELISA test was run using standard procedures with a 1/500 dilution of primary (anti-42-kDa) ascites fluid and a 1/2000 dilution of horseradish peroxidase-conjugated goat anti-mouse antibody (EIA grade, Bio-Rad).

Western blots were run according to [8], after separation of the proteins present in the CHAPS supernatant by SDS-PAGE. The proteins transferred to the nitrocellulose sheet were stained for 10 min in a solution containing 3% (w/w) trichloracetic acid and 0.2% (w/w) Ponceau red. Proteins were assayed according to [16].

2.4. *Reconstitution experiments*

Reconstitution was carried out following a procedure modified from [17]. Soybean asolectin (Sigma IV-S) was dissolved in chloroform at a concentration of 200 mg/ml and stored at −20°C under N_2. To prepare lipids for use during reconstitution, 4.4 mg asolectin was dried under N_2, rotating the tube to form a thin film. To remove residual chloroform, the phospholipids were washed with 1 ml of cold (−20°C) diethylether and evaporated to dryness. The lipids were dried for an additional 30 min under vacuum to remove all traces of solvent. The lipids were resuspended in 400 μl of solution containing 50 mM potassium phosphate (pH 7.5) and 1 mM DTT. The mixture was bath-sonicated (Bransonic-5 sonicator) to clarity (about 30 min) in a tube immersed in ice. CHAPS (1%, final concentration) was added from a 10% stock solution prepared in 50 mM potassium (pH 7.5), and the tube was vortexed for 15 s. Glycerol (20%, final concentration, v/v) was added and the tube was vortexed again for 15 s. One mg of membrane protein (1.7 ml), either from 'CHAPS supernatant' or from the 120-kDa fraction (as referred to in section 3) were added to the tube, and incubated for 30 min at 4°C. The insoluble material was then removed by centrifugation at 100000 × *g* for 45 min. Reconstitution was done by adding 2 ml of the supernatant in a tube containing 5 mg dried asolectin and 178 μl of 50 mM potassium phosphate buffer (pH 7.5). The tube was sonicated for 30 min, and 44 μl of a 10% CHAPS solution were added before vortexing, and incubation for 30 min at 4°C. Proteoliposomes were formed by rapid injection (Pasteur pipet) of the ice-cold solubilized protein/phospholipid mixture into 25 ml of 50 mM potassium phosphate (pH 7.5). The tube was incubated for 20 min at room temperature and centrifuged for 1 h at 100000 × *g*. The final pellet was resuspended into 100 μl potassium phosphate buffer (pH 7.5).

2.5. *Uptake experiments*

Uptake experiments with reconstituted proteoliposomes containing either the 'CHAPS supernatant' or the '120-kDa fraction' were run according to the technique previously designed to study active uptake by native plasma membrane vesicles [9], after imposition of pH (ΔpH) and electrical ($\Delta\psi$) gradients across the membrane. For comparison, uptake experiments were also run with native plasma membrane vesicles.

All experiments (HPLC and uptake experiments) were repeated at least three times with similar results. In each uptake experiment, data points were the mean of 4 independent samples.

3. RESULTS AND DISCUSSION

3.1. *Specificity of the anti-42-kDa serum*

The specificity of the serum used in this paper was tested by immunoblotting. The blots obtained after separation of total plasma membrane proteins and transfer to nitrocellulose were probed successively with anti-42-kDa ascites and with peroxidase-labeled anti-mouse IgG. The ascites fluid reacted with a single, rather wide region around 42 kDa (Fig. 1). Various polyclonal anti-42-kDa sera, including the ascites fluid used for this study, inhibited active uptake of sucrose into protoplasts [8] and into purified plasma membrane vesicles ([18]; O. Gallet, R. Lemoine, C. Gaillard, C. Larsson and S. Delrot, submitted).

3.2. *Fractionation of membrane proteins*

After gel filtration under non-denaturating conditions, a broad region around 120 kDa was the most reactive in ELISA with the anti-42-kDa serum (Fig. 2A). This peak accounted for about 23% of the total plasma membrane proteins. When the 120-kDa peak was recovered, denaturated by SDS (1%, 80°C for

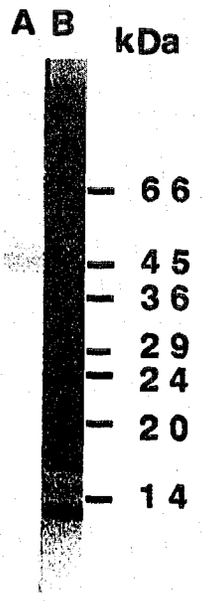

Fig. 1. The specificity of the antiserum used for ELISA detection after HPLC of CHAPS-soluble proteins. Plasma membrane proteins were separated by SDS-PAGE, blotted onto a nitrocellulose sheet and incubated with a mouse polyclonal serum directed against the 42-kDa protein of the plasma membrane. (A) Immunoblot; (B) polypeptides as revealed by Ponceau red, after transfer to the nitrocellulose sheet. The position of the molecular weight standards is indicated on the right of the figure.

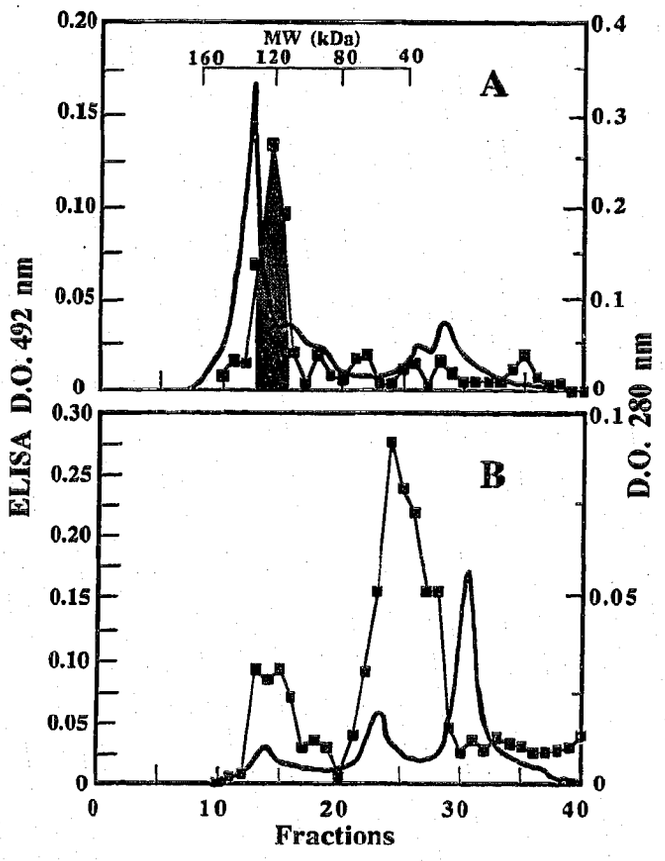

Fig. 2. ELISA detection of CHAPS-soluble proteins after separation on a gel filtration column. (A) The proteins solubilized by CHAPS were applied to a TSK SW-3000 column, and the ability of the eluted fractions to react with anti-42-kDa serum was monitored by ELISA (■). Continuous line, absorbance at 280 nm. Dextran blue 2000, used as a marker of void volume, was eluted in fraction 10. (B) The 120 kDa region (dotted area) from Fig. 2A was recovered and denatured by SDS before gel filtraiton.

15 min) and resubmitted to gel filtration, a major ELISA-reactive peak was observed around 40 kDa, while the 120-kDa peak was strongly reduced (Fig. 2B) or completely disappeared, depending on the experiments. The ELISA data were paralleled by data obtained with plasma membranes double-labeled with [^{3}H]NEM/[^{14}C]NEM according to a procedure [7] allowing the differential labeling of the polypeptides recognizing sucrose. Indeed, the peak of differential label which was located at around 120 kDa in HPLC eluates from non-denaturated membranes switched to 40 kDa after denaturation by SDS (data not shown).

These data show that the 42-kDa polypeptide previously identified as a putative sucrose carrier may oligomerize or may be associated with other polypeptides, at least under the conditions used for these experiments in vitro. Although trimerization of transport proteins has already been reported for bacteria [19], more work is needed to conclude on the nature of the

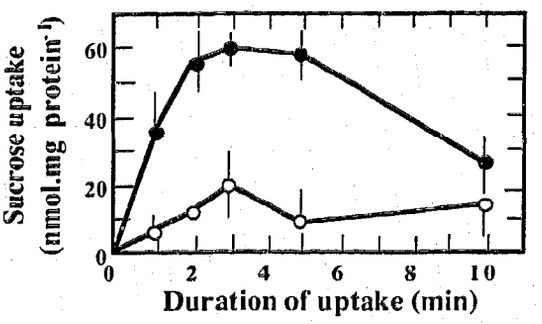

Fig. 3. Time course of uptake of 1 mM sucrose by proteoliposomes reconstituted with the 120-kDa fraction. Experiments were run in the presence (●), or in the absence (○) of imposed $\Delta pH + \Delta\psi$.

macromolecular structure in which the 42-kDa polypeptide is associated.

3.3. *Active uptake by reconstituted proteoliposomes*

The 120-kDa fraction, recovered after non-denaturing gel filtration of the plasma membrane polypeptides, was used in order to reconstitute sucrose transport activity into proteoliposomes. Active uptake of sucrose [9–11] or of amino acids [20] can be driven by imposing ionic gradients to sugar beet plasma membrane vesicles. When the 120-kDa fraction was used for reconstitution experiments, about 60% of the fraction was reincorporated into the proteoliposomes. In the absence of energization, little sucrose was retained in the reconstituted proteoliposomes (Fig. 3, lower curve). A proton-motive force-driven sucrose uptake was obtained after imposition of a pH gradient + an electrical gradient (Fig. 3, upper curve). The difference between the energized conditions and the non-energized conditions is representative of an active, proton-driven uptake of sucrose. No active uptake of valine could be observed with the same preparations, since there was no difference between energized and non-energized conditions (Fig. 4). The amounts of valine retained passively on the vesicles were higher than the amounts of sucrose retained passively, possibly due to the adsorption of

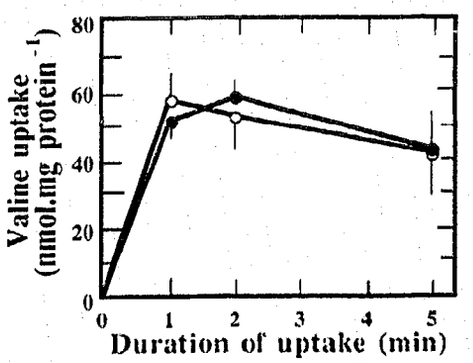

Fig. 4. Time-dependent uptake of 1 mM valine in the presence (●) or in the absence (○) of imposed $\Delta pH + \Delta\psi$.

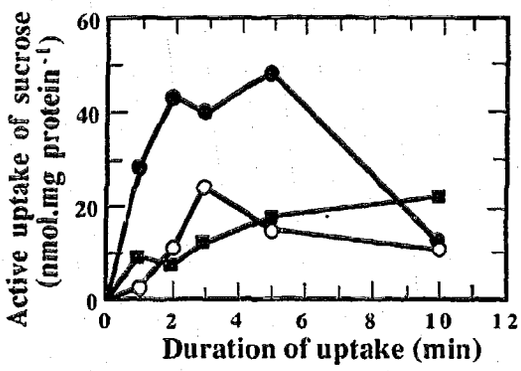

Fig. 5. Active transport of sucrose into native plasma membrane vesicles (■) and into proteoliposomes reconstituted with the CHAPS supernatant (○) or the 120-kDa fraction (●).

charged valine with the membrane proteins. Comparison of Figs. 3 and 4 shows that the reconstituted active uptake system was specific for sucrose. After 2 min incubation, active uptake of sucrose was completely abolished in the presence of 0.1 mM $HgCl_2$, and was inhibited to 20% of the control value in the presence of 0.5 mM NEM.

The time course of active uptake of sucrose into native plasma membrane vesicles, into proteoliposomes made from the CHAPS supernatant and into proteoliposomes made from the 120-kDa fraction was compared (Fig. 5). Proteoliposomes made from the CHAPS supernatant did not show a stronger accumulation than the native plasma membranes. Yet, proteoliposomes made from the 120-kDa fraction accumulate more sucrose than native plasma membranes. Also, the initial rate of influx was much higher in these proteoliposomes than in the native plasma membranes (Fig. 5).

In conclusion, the paper presents the first data concerning the reconstitution of a carrier mediating proton-driven uptake of an organic solute at the plasma membrane. The reconstituted transport activity was sensitive to thiol reagents, was specific for sucrose, and the specific activity of transport (nmol sucrose/mg protein/min) was higher in the reconstituted system than in native plasma membranes. The methods designed in this study should prove useful to monitor the purification of the sucrose carrier, and possibly of other proton-driven carriers of the plasmalemma.

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